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ORIGINAL ARTICLE

Localization and Up-Regulation of Cysteinyl Leukotriene-2 Receptor in Human Allergic Nasal Mucosa

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ABSTRACT

Background: Cysteinyl leukotrienes (CysLTs) are lipid mediators that have been implicated in the pathogenesis of allergic rhinitis. Pharmacological studies using CysLTs indicate that 2 classes of receptors exist, namely, $CysLT_1$ and $CysLT_2$ receptors. The former class of receptors is sensitive to the $CysLT_1$ antagonists currently used to treat asthma and allergic rhinitis, and its localization has been previously examined by our group using immunohistochemistry and in situ hybridization techniques. We investigated the expression and localization of the $CysLT_2$ receptor in human nasal mucosa by western blot and immunohistochemical analyses.

Methods: Human turbinates were obtained after turbinectomy from 16 patients with nasal obstruction refractory to medical therapy. To identify the cells expressing the $CysLT_2$ receptor, double immunostaining was performed by using anti-$CysLT_2$ receptor antibody and anti-CD31 (endothelial cell) antibody or anti-smooth muscle actin antibody.

Results: A 39 kDa band was detected on the western blots of human turbinates samples by using the anti-$CysLT_2$ receptor antibody. The expression level of the $CysLT_2$ receptor in patients with nasal allergy was higher than that in patients with non-allergic rhinitis. The immunohistochemical study also showed an intense immunoreactivity for $CysLT_2$ receptor in both vascular endothelial cells and vascular smooth muscles.

Conclusions: The results indicated that the $CysLT_2$ receptor plays a primary role in the vascular responses in the upper respiratory tract.

KEY WORDS

allergic rhinitis, $CysLT_2$ receptor, immunohistochemistry, Western blot

INTRODUCTION

The allergic response is a complex process involving the interaction of many mediators. Among these mediators, cysteinyl leleukotrienes (CysLTs) are important for the pathogenesis of airway allergic diseases such as allergic rhinitis and asthma.[1] Pharmacological studies using CysLTs indicate that at least 2 classes of receptors exist, namely $CysLT_1$ and $CysLT_2$ receptors.[2,3] Our previous immunohistochemical study showed that anti-$CysLT_1$ receptor antibody labeled eosinophils, mast cells, macrophages, neutrophils, and vascular endothelial cells in human nasal mucosa,[4] suggesting that in allergic rhinitis, the major targets of the $CysLT_1$ antagonists are vascular bed and infiltrated leukocytes.

While the $CysLT_1$ receptor has been studied in great detail in relation to allergic rhinitis and asthma,[1] our knowledge of the $CysLT_2$ receptor function is still rather limited, which is in part because of the lack of a specific receptor antagonist. To understand the role of the $CysLT_2$ receptor in nasal allergy, it is very important to gather information about the localization of the receptor. However, few studies have shown the $CysLT_2$ receptor expression in nasal specimens, specifically in epithelial cells,[5] submucosal glands,[6] and inflmammatory leukocytes.[5-7] In the present study, we performed immunohistochemical analysis on sur-

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Table 1 Demographic characteristics of allergic and non-allergic patients

	Allergic rhinitis $N = 10$	Non-allergic patients $N = 6$	p Value
Sex (male/female)	4/6	3/3	NS
Age	34 ± 16	38 ± 10	NS
Specific IgE to house dust mite (d1) (kU/L)	5.3 ± 4.6	<0.35	0.004
Total IgE (kU/L)	331 ± 297	100 ± 61	NS
Blood eosinophils (cells/μL)	441 ± 296	137 ± 57	0.04
Local eosinophils (cells/mm2)	58.2 ± 25.6	9.2 ± 10.7	0.003
Total nasal symptom score (TNSS)	5.8 ± 2.8	2.3 ± 1.0	0.03
Nasal obstruction score	3.2 ± 0.8	1.7 ± 0.8	0.02
Sneezing score	1.7 ± 1.4	0.5 ± 0.5	NS
Rhinorrhea score	1.0 ± 1.3	0.2 ± 0.4	NS

Data expressed as mean ± SD or No. NS, not significant.

gical specimens to demonstrate the localization of $CysLT_2$ receptor in nasal structural cells. The western blot analysis also confirmed the up-regulation of the $CysLT_2$ receptor in perennial allergic nasal mucosa.

METHODS

TISSUE PREPARATION

Human inferior turbinates were obtained after turbinectomy from 16 patients with nasal obstruction refractory to medical therapy. Informed consent was obtained from all patients, and this study was approved by the ethics committee of Sapporo Medical University (approval number: 24-14). All patients were nonsmokers, of which 10 had perennial allergy to mites as diagnosed by questionnaires and the CAP test (Pharmacia, Uppsala, Sweden). The remaining 6 patients who had concomitant septoplasty because of symptomatic nasal septal deviation, with a negative CAP test for major inhaled allergens such as mites, birch, grass, mugwort and Japanese cedar were included in non-allergic rhinitis group as control. Severity of nasal symptoms (nasal obstruction, sneezing and rhinorrhea) was assessed on 0-4 category scale and Total Nasal Symptom Score (TNSS) was calculated. All medications, including antibiotics, were prohibited for at least 3 weeks prior to the study. Demographic and clinical characteristics of the patients are summarized in Table 1. The nasal mucosal specimens dissected from the cartilage, were either frozen immediately in liquid nitrogen and stored at -70℃ for protein extraction or fixed in 10% formalin for immunohistochemistry.

WESTERN BLOT ANALYSIS

The nasal samples were homogenised in the Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with the Proteiase Inhibitor Cocktail (Sigma, St. Louis, MO, USA). Protein extracts were then purified using the PAGEprep™ Advance Kit (Thermo Fisher Scientific), and protein concentrations in the homogenates were determined by the BCA Protein Assay Kit (Thermo Fisher Scientific). Protein extracts (20 μg of protein each sample) were separated in a 4-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA, USA). Membranes were subsequently blocked and probed with a rabbit polyclonal anti-human $CysLT_2$ antibody (1 : 500; ab75160, Abcam, Cambridge, UK). A rabbit polyclonal anti-human antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also used as an internal control. The bands were visualized using WesternBreeeze® Chromogenic Immunodetection Kit (Invitrogen). The ratio of the protein levels of the $CysLT_2$ receptor to GAPDH was determined by densitometric analysis.

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Deparaffinized sections were initially incubated with 3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. After microwave treatment (10 min at 500 W in citrate buffer), the sections were incubated in blocking solution (10% normal goat serum in PBS) for 30 min before overnight incubation with the primary antibody at 4℃. A rabbit polyclonal anti-human $CysLT_2$ antibody raised against a peptide derived from the first extracellular domain of the human $CysLT_2$ receptor (ab115453, Abcam) was used as the primary antibody at a concentration of 10 μg/ml. The sections were washed, and incubated for 30 min with EnVision$^+$, Peroxidase (Dako, Carpinteria, CA, USA). A further wash in PBS was followed by developing in DAB (Dako) as a chromogen for signal visualization. The slides were counterstained with Mayer's haematoxylin and mounted on a cover slip using a mounting medium.

To identify the subsets of cells expressing $CysLT_2$ receptor, the following monoclonal antibodies were

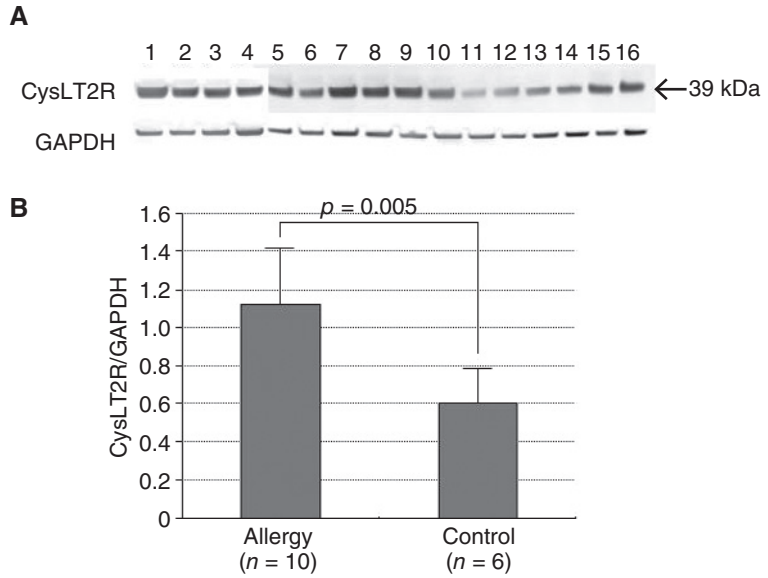

Fig. 1 **A**: Western blots showing CysLT2 receptor protein level in allergic nasal mucosa (lane 1-10) and nonallergic nasal mucosa (lane11-16). The antibody recognized a single, 39-kDa protein -the CysLT2 receptor. **B**: Densitometric analysis of the relative protein level of CysLT2 receptor as shown. The data were expressed as mean + SD and normalized to GAPDH.

used: anti-CD31 (JC70A clone, Dako) for vascular endothelial cells, anti-smooth muscle actin (1A4 clone, Dako) for vascular smooth muscle, and anti-human CD45 (2B11 + PD7/26 clone, Dako) for leukocyte common antigen. For double staining, deparaffinized sections were incubated overnight at 4℃ with a combination of rabbit polyclonal anti-human $CysLT_2$ antibody and one of mouse monoclonal anti-human phenotypical maker antibody.

The sections were washed in PBS and incubated for 30 min with both Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1 : 50; Molecular Probes, OR, USA). The sections were mounted using Vectashield® mounting medium with 4',6 diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and examined under an Olympus BX51 microscope fitted with a DP70 CCD camera (Olympus Optical, Tokyo, Japan). All image analysis was performed with DP Controller and DP Manager Software (Olympus Optical). Using this method, the $CysLT_2$ receptor-expressing cells were visualized as red, vascular endothelial cells were green, and the combined signal was visualized as yellow.

STATISTICAL ANALYSIS

The protein levels of the $CysLT_2$ receptor and GAPDH detected in the duplicate experiment of western blot analysis were determined, and data were presented as mean ± standard deviation. The non-parametric Mann-Whitney U test was used to evaluate differences between perennial allergic rhinitis and control subjects. Values of $P \leq 0.05$ were considered to be statistically significant.

RESULTS

WESTERN BLOTTING

To determine the protein size of the $CysLT_2$ receptor in human nasal mucosa, its expression was examined by using western blot analysis. As shown in Figure 1 A, a single band of the predicted molecular weight (approximately 39 kDa) was detected. Densitometric quantification of the bands was performed to determine the expression levels of the $CysLT_2$ receptor and GAPDH in allergic and non-allergic nasal mucosa (Fig. 1B). The ratio of $CysLT_2$ receptor/GAPDH in nasal mucosa from patients with nasal allergy was significantly higher than that from non-allergic patients (allergic rhinitis: 1.12 ± 0.29, control: 0.60 ± 0.16, p = 0.005). No significant correlation was found between the level of $CysLT_2$ receptor and nasal symptom score, total and specific IgE, local and blood eosinophil number (data not shown).

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The distribution of the $CysLT_2$ receptor in nasal mucosa was examined by means of immunohistochemistry. As shown in Figure 2A, a significant immunoreactivity for the $CysLT_2$ receptor was detected in blood vessels. In contrast, epithelial cells and submucosal

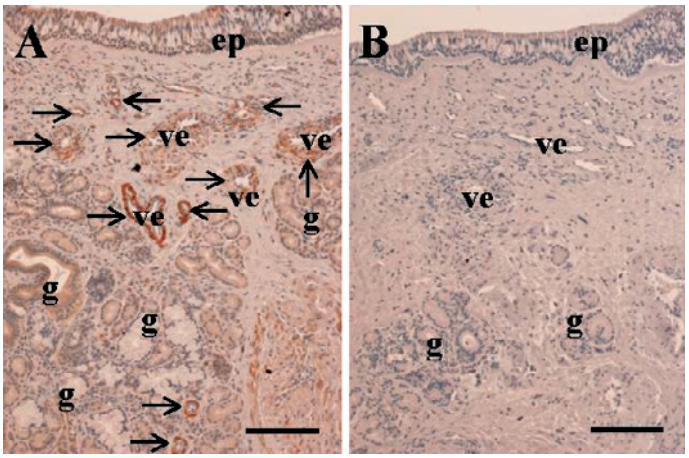

Fig. 2 Immunohistochemical staining for CysLT2 receptor in human nasal mucosa. Inferior turbinates were stained with anti-human CysLT2 receptor antibody (**A**) or normal rabbit immunoglobulin (**B**). CysLT2 receptor immunoreactivity was observed mainly on blood vessels (arrow). ep, epithelial cells; g, submucosal glands; v, blood vessels. Scale bar = 200 μm.

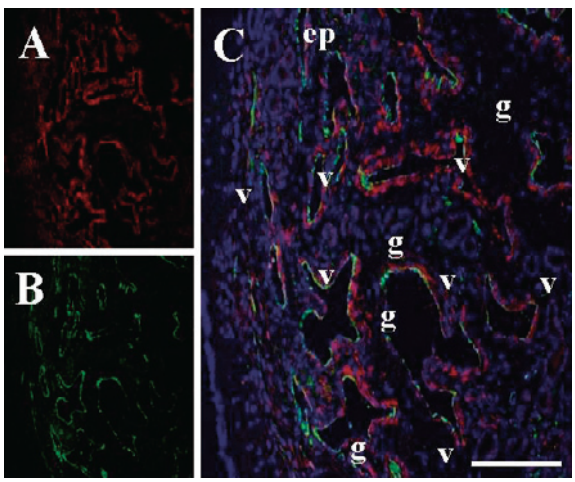

Fig. 3 Identification of CysLT2 receptor on blood vessels of the nasal mucosa. (**A**): CysLT2 receptor positive cells. (**B**): Anti-CD31 stain as an identification marker for vascular endothelial cells. (**C**): Overlay image. CysLT2 protein (red) shows co-localization with anti-CD31 antibody (green) and the combined signal is visualized as yellow. Not only vascular endothelial cells, but also vascular smooth muscles express CysLT2 receptor. ep, epithelial cells; g, submucosal glands; v, blood vessels. Scale bar = 200 μm.

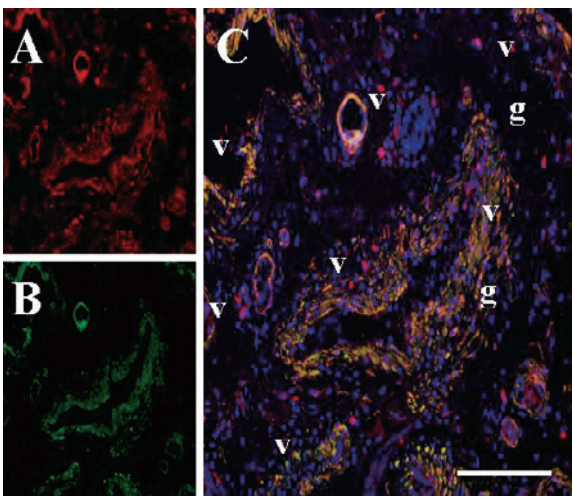

Fig. 4 Identification of CysLT2 receptor on vascular smooth muscle of the nasal mucosa. (**A**): CysLT2 receptor positive cells. (**B**): Anti-smooth muscle actin stain as an identification marker for vascular smooth muscle. (**C**): Overlay image. CysLT2 protein (red) shows co-localization with anti-smooth muscle actin antibody (green) and the combined signal is visualized as yellow. Vascular smooth muscles express CysLT2 receptor. g, submucosal glands; v, blood vessels. Scale bar = 100 μm.

glands showed little or no expression. Specificity of the staining was confirmed by the absence of labeling with normal rabbit immunoglobulin (Fig. 2B). To further clarify the vascular cell types expressing the $CysLT_2$ receptor, double immunofluorescence staining was performed for $CysLT_2$ receptor (Fig. 3A, 4A) and for CD31 and smooth muscle actin, which are as a vascular endothelial cell marker (Fig. 3B) and a vascular smooth muscle marker (Fig. 4B), respectively. As shown in Figure 3C, 4C, not only vascular endothelial cells but also vascular smooth muscle showed intense immunoreactivity for the $CysLT_2$ receptor. In

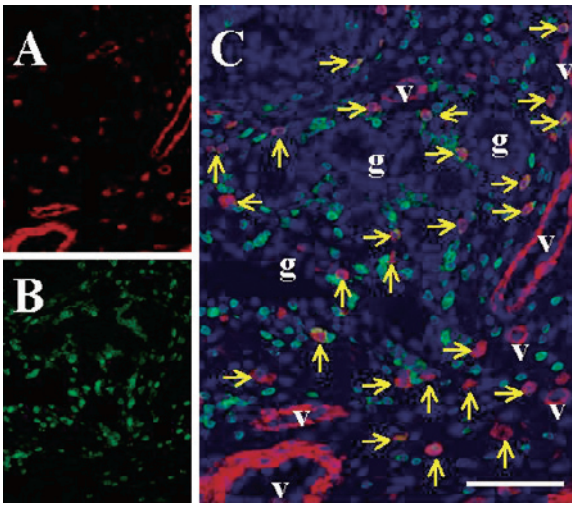

Fig. 5 Identification of CysLT2 receptor on inflammatory leukocytes in the nasal mucosa. (**A**): CysLT2 receptor positive cells. (**B**): Anti-CD45 stain as an identification for leukocyte common antigen. (**C**): Overlay image. CysLT2 protein (red) shows co-localization with anti-CD45 antibody (green) and the combined signal is visualized as yellow. Not only blood vessels, but also some inflammatory leukocytes (arrow) express CysLT2 receptor. g, submucosal glands; v, blood vessels. Scale bar = 100 μm.

order to clarify the $CysLT_2$ receptor on inflammatory leukocytes, we performed double immunofluorescence staining for $CysLT_2$ receptor (Fig. 5A) and leukocyte common antigen (Fig. 5B). As shown in Figure 5C, some inflammatory leukocytes express $CysLT_2$ receptor. The further double immunohistochemical staining revealed that the majority of these $CysLT_2$ receptor positive cells were mast cells and macrophages (data not shown). The pattern of the immunohistochemical findings for all 16 patients were remarkably similar.

DISCUSSION

The results from this study, obtained nasal surgical specimens, indicate that the nasal $CysLT_2$ receptor localizes exclusively in blood vessels. In the past, only few studies have been designed to explore the expression of the $CysLT_2$ receptor in nasal mucosa biopsies from patients with allergic rhinitis.[5-7] Corrigan *et al.* showed the presence of the $CysLT_2$ receptor in inflammatory leukocytes, mucus glands and epithelial cells in nasal biopsies from patients with chronic rhinosinusitis.[5] On the other hand, the $CysLT_2$ receptor immunoreactivity in sinus mucosa obtained from endoscopic sinus surgery was mainly detected in inflammatory leukocytes.[6,7] These previous reports did not examine the expression of the $CysLT_2$ receptor in blood vessels.[5-7] To the best of our knowledge, our study shows for the first time, the presence of $CysLT_2$ in human nasal blood vessels.

The studies using mice with deletions[8] or overexpression[9] of the $CysLT_2$ receptor in the lungs have suggested a prominent role for the receptor in mediating vascular permeability. In vitro, human umbilical vein endothelial cells (HUVECs) express the $CysLT_2$ receptor mRNA in vast excess (>4000-fold) of the $CysLT_1$ receptor mRNA, and Ca^{2+} fluxes caused by CysLTs in HUVECs may result from perturbation of the $CysLT_2$ receptor rather than the expected $CysLT_1$ receptor.[10] Therefore, the $CysLT_2$ receptor-mediated nasal vascular response may affect nasal vascular permeability and consequent plasma leakage may be cause local tissue edema and watery rhinorrhea. Moreover, CysLTs have been found to elicit several specific functional responses in endothelial cells, eg, the synthesis of platelet activating factor,[11] secretion of von Willebrand factor, and surface expression of P-selectin.[12] These effects may be in part due to activation of the $CysLT_2$ receptor.

Our present immunohistochemical studies have clearly shown the expression of the $CysLT_2$ receptor not only in vascular endothelial cells, but also in vascular smooth muscle cells. With regards to the expression of the $CysLT_2$ receptor in human vascular smooth muscle cells, only the presence of its mRNA in coronary artery has been reported using RT-PCR and in situ hybridization.[13] When human coronary smooth muscle cells are stimulated with LTC_4, the intracellular calcium concentration increases, which suggests that the contraction induced by LTC_4 may be mediated by the $CysLT_2$ receptor.[13] In contrast, activation of the $CysLT_2$ receptor in endothelial cells leads the release of a relaxant factor, nitric oxide (NO).[14] Because no data on the experimental model of allergic rhinitis using $CysLT_2$ receptor-deficient mice or $CysLT_2$ receptor antagonists, the biological significance of the $CysLT_2$ receptor in nasal vasculature remains unclear.

The expression of $CysLT_2$ receptor was found not only on nasal vasculature, but also on some infiltrated leukocytes such as mast cells and macrophages in the nasal mucosa. It has been reported that mast cells, macrophages and eosinophils in nasal lavage from patients with active seasonal allergic rhinitis expressed $CysLT_2$ receptor.[15] The roles of $CysLT_2$ receptor in these inflammatory leukocytes still remain unclear.

Our present western blot analysis has showed that the expression of the $CysLT_2$ receptor increased significantly in allergic nasal mucosa compared to non-allergic samples. Previous studies on paranasal sinus mucosa have also shown that the $CysLT_2$ receptor mRNA expression in chronic rhinosinusitis was significantly higher than that in controls.[6,7] In addition, mucosal hyper-responsiveness, a pivotal pathophysiological characteristic of allergic rhinitis has been observed in patients with perennial allergic rhinitis.[15] It is therefore, logical to hypothesize that hyperrespon-

siveness might stem from the up-regulation of the leukotriene receptors. The $CysLT_2$ receptor expression is subjected to regulation in vitro. Our previous study showed that CysLTs and Th2 cytokines such as IL-13 enhanced the expression of $CysLT_2$ receptor in human monocytes in vitro.[16] Some studies have also demonstrated increased local levels of IL-4 and IL-13 following allergen provocation in allergic rhinitis.[17] Therefore, it is possible that the $CysLT_2$ receptor in allergic airways may be up-regulated by IL-13 released from leukocytes.

To further clarify the role of CysLTs in the allergic airways, a dual $CysLT_1$/$CysLT_2$ receptor antagonist might be more appreciate. However, the pharmacology of the $CysLT_2$ receptor is less defined than that of $CysLT_1$ receptor, mainly because of the lack of a selective antagonist. The dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 may act as a $CysLT_1$ receptor agonist.[18] Thus, the clinical advantages of dual $CysLT_1$ and $CysLT_2$ receptor antagonists for the treatment of allergic rhinitis are still unknown.

In conclusion, our results indicate that the $CysLT_2$ receptor plays a primary role in the vascular responses in the upper respiratory tract. The present findings should be of considerable interest for elucidating the potential roles of the $CysLT_2$ receptor in upper airway diseases such as allergic rhinitis.

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